Title: Use of Quantitative Polymerase Chain Reaction (qPCR) for the Diagnosis and Monitoring of CNS Leukaemia

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Main text:

Despite the excellent overall survival in childhood acute lymphoblastic leukemia (ALL), central nervous system (CNS) disease continues to pose challenges. Currently, only 3-5% children with ALL present with cytological evidence of CNS involvement [1], however CNS-directed therapy is essential for cure, and the majority of CNS relapses occur in children who had negative CNS cytology at diagnosis [2]. This implies that the frequency of patients with CNS involvement may be higher than currently detected. There is a clear need for improving diagnostic accuracy of CNS involvement. Leukemic cells, being clonal in origin, carry VDJ gene rearrangements unique to individual patients. Using allele-specific oligonucleotides (ASO) primers TaqMan qPCR accurately estimates minimal residual disease (MRD) and predicts bone marrow relapse. [3]. However, bone marrow MRD status does not predict the risk of CNS relapse.

We investigated if qPCR could identify patients with submicroscopic levels of CNS involvement at diagnosis and to track therapy response. Our research questions were: i) can amplifiable DNA be extracted from leftover cerebrospinal fluid (CSF) samples obtained for routine CSF cytology? ii) Can allele-specific PCR primers designed for bone marrow MRD detect leukemic in the CNS? Iii) Is CSF qPCR able to track treatment response in the CNS?

The study was approved by the West of Scotland Research Ethics Committee (WoSREC reference: 09/SO703/77). Between July 2011 and July 2014, children (1-18 years old) diagnosed with ALL at the Royal Hospital for Sick Children, Glasgow were enrolled in the study. Diagnostic and follow-up CSF samples during induction therapy were collected. DNA was extracted using Qiagen QIAmp DNA Micro kit and quantified using TaqMan Real-Time qPCR assay for Albumin [4]. ASO primers were designed at the Glasgow MRD laboratory according to Biomed-II guidelines [5].

During the study period, 57 patients were enrolled. Out of these 19 were excluded from analysis (see supplementary table 1 for details). This left samples from 38 patients for analysis (31 patients with CNS-1 disease (no blasts in CSF cytology), 5 with TLP+ve

(traumatic lumbar puncture with identifiable blasts) and 2 with TLP-ve (traumatic lumbar puncture without blasts)) (supplementary table 2 and 3).

Real-time qPCR revealed detectable leukemic DNA in 15/38 patients (39.5%) (designated qPCR positive). Among these, 11 were CNS-1 patients and 4 were TLP+ve patients. 1/5 TLP+ve and 2/2 TLP-ve patients were CSF qPCR negative. Comparison of CSF qPCR positive *vs.* negative patients showed an association with a higher WCC (mean 26.6 x 10^9/l vs 19.4 x 10^9/l) and high-risk cytogenetics (p=0.04) – factors recognized as high-risk for CNS relapse. Three out of 4 patients with T-ALL were CSF qPCR positive (table 1).

Next, serial samples from 18 patients during the induction phase of treatment were analysed (11 patients were CSF qPCR negative and 7 were CSF qPCR positive at diagnosis). All patients were CSF qPCR negative on day 29 (end of induction). Two day 8 samples (patient #22 and #37), and one day 15 sample (patient #37) were CSF qPCR positive. (figure 1). Overall, these results indicate that qPCR positive disease is rapidly cleared from the CSF during induction.

Twenty patients had sufficient template DNA to test two primer/probe sets, 13 patients were negative and 4 patients were positive with both sets, whilst 3 patients were positive with one set but negative with the other (supplementary figure 1).

CSF qPCR positivity at diagnosis was not associated with BM MRD risk status at the end of induction. In this small study, no CNS relapses were seen during the period of follow up (71 ± 10 months, median ±SD). One CSF qPCR negative patient suffered isolated BM relapse at 4-years (Supplementary table 3).

Our findings indicate that the use of qPCR detects occult CNS involvement in more than one-third of children with acute lymphoblastic leukemia. This is consistent with other newer methods such as flow-cytometry [6]. However, this technique also has important limitations. Firstly, 19/57 (33%) patient samples could not be used for ASO qPCR testing due to lack of amplifiable DNA or suitable primers. Some of these patients may represent true negative

cases with no circulating blasts in the CSF, alternatively the small volume of CSF obtained may have yielded inadequate DNA. Secondly, 1/5 TLP+ patients was negative by qPCR although this may reflect either false positive cytology or false negative qPCR. Thirdly, 3/20 patients had discordant results when testing two primer/probe sets. This is most likely to be due to differing sensitivity of the two assays. Alternatively, there could be subclonal selection of cells bearing only one of the two VDJ rearrangements in the CNS. A body of experimental evidence from our previous work [7], and others [8] argue against this. Finally, the real power of developing biomarkers of CNS leukemia would be the ability to track response to treatment within the CNS compartment. Thus, an ideal test should be able to separate patients into rapid and slow responders, with subsequent follow-up needed to confirm that this has prognostic significance. In our study, all patient samples became negative by the end of induction chemotherapy, suggesting that it is too insensitive for disease response assessment.

Given these limitations, qPCR of CSF is unlikely to be clinically useful. Alternative strategies such as flow-cytometry or NGS still rely on detection of circulating cells within the CSF. Historical reports of autopsies [9] and our previous data [10] show that leukemic blasts are often embedded in the meninges and not present in a free-floating state. Future research into soluble biomarkers released by leukemic cells into circulating CSF might be the best way forward.

Acknowledgements

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Contributions

1. Yasar Mehmood Yousafzai: Performed research, analysed data and wrote manuscript. 2. Linda Smith: Contributed to essential reagents and tools, analysed data, and approved manuscript 3.

Amanda Smith: Contributed to essential reagents and tools, analysed data, and approved manuscript 4. Saeeda Bhatti: Contributed to essential reagents and tools, analysed data, and approved manuscript 5. Marry Gardiner: Contributed to essential reagents and tools, analysed data, and approved manuscript 6. Anthony Cousins: Contributed to essential reagents and tools, analysed data, and approved manuscript 7. Frances Fee: Contributed to essential reagents and tools, analysed data, and approved manuscript 8. Sandra Chudleigh: Contributed to essential reagents and tools, analysed data, and approved manuscript 9. Alison Spence: Contributed to patient recruitment, analysed data, and approved manuscript 10. Wendy Taylor: Contributed to patient recruitment, analysed data, and approved manuscript 11. Nicholas Heaney: Contributed to patient recruitment, performed laboratory analysis of samples, and approved manuscript 12. Brenda Gibson: Contributed to patient recruitment, performed critical analysis of research and approved manuscript 13. Gerry Graham: Performed critical analysis of research and approved manuscript 14. Chris Halsey: Conceived research, performed research, wrote manuscript.

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Figure

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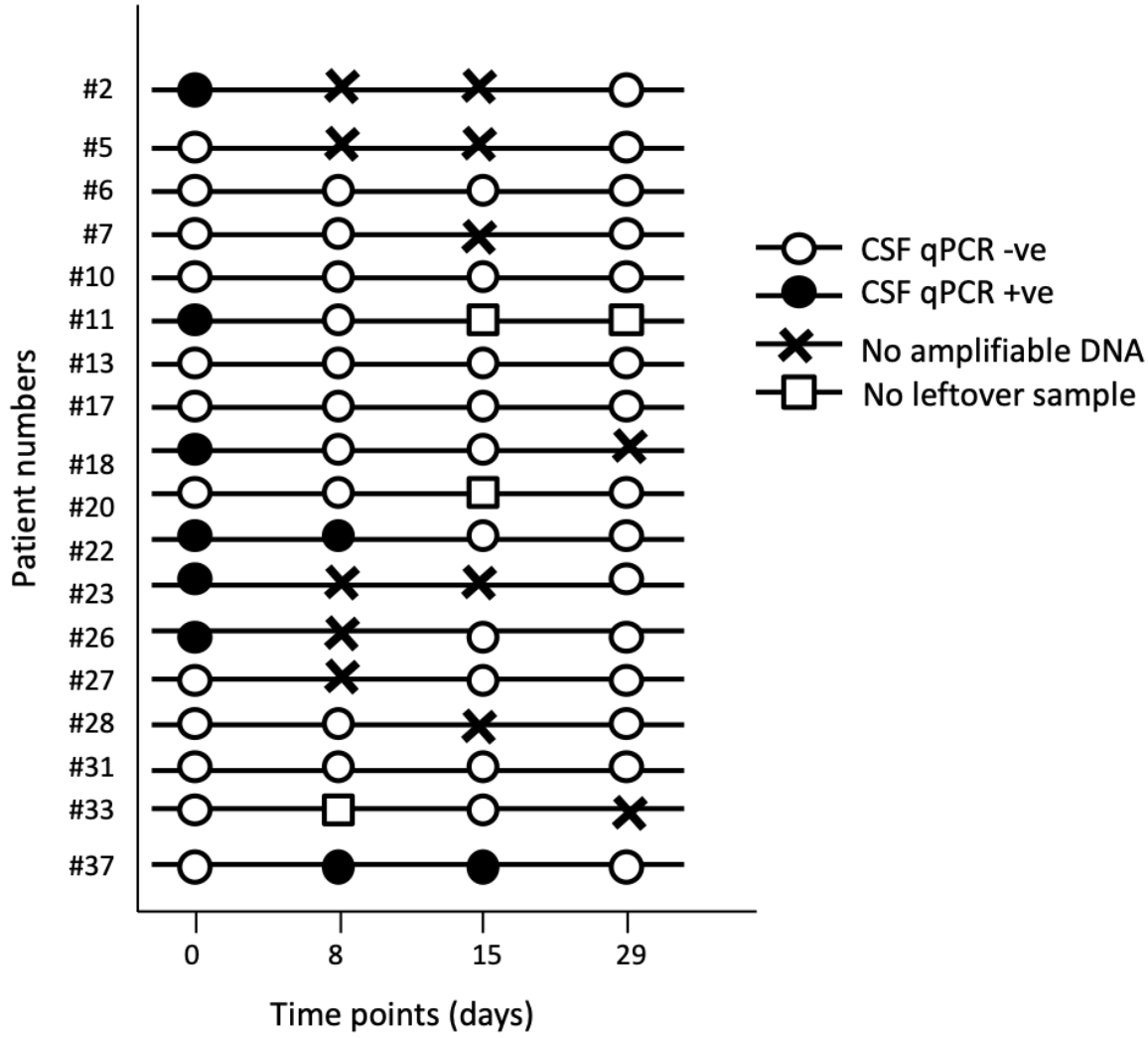

Figure Legend

Figure 1: qPCR positivity of representative set of patients at diagnosis and during induction period. Time points: Days post diagnosis, open circles = qPCR negative, closed circles = qPCR positive, cross= No amplifiable DNA, open box = no leftover samples available.

Table 1

Variable	Category	QPCR+ve		QPCR-ve		
		n	%	n	%	
		15	**39.5%**	**23**	**60.5%**	**p-value***
Age at diagnosis	<10 year	12	39%	19	61%	0.839
	>10 years	3	43%	4	57%	
Sex	Male	12	50%	12	50%	0.101
	Female	3	21%	11	79%	
Immunophenotype	BCP-ALL	12	35%	22	65%	0.280
	T-ALL	3	75%	1	25%	
WCC	<10	4	21%	15	79%	0.043
	10-50	9	64.3%	5	35.7%	
	>50	2	40%	3	60%	
CNS status	CNS-1	11	35%	20	65%	0.084
	TLP+	4	80%	1	20%	
	TLP-	0	0%	2	100%	
Cytogenetic risk✞	Low risk	7	28%	18	72%	0.04
	High risk	3	100%	0	0%	
	Others	5	50%	5	50%	
Day 29 MRD	MRD risk	8	50%	8	50%	0.258
	Low risk	7	35%	15	65%	

Table Legend 1

Table 1 Comparison between CSF qPCR +ve and qPCR –ve patients

*calculated using Chi squared test

✞cytogenetic risk:

Low risk	t(12;21), High hyperdiploid
High risk	t(9;22), *MLL*-rearranged
Others	t(7;9), del 12p, t(9;18), complex cytogenetics of uncertain origin

Acknowledgements:

1. Yasar Mehmood Yousafzai: Performed research, analysed data and wrote manuscript
2. Linda Smith: Contributed to essential reagents and tools, analysed data, and approved manuscript
3. Amanda Smith: Contributed to essential reagents and tools, analysed data, and approved manuscript
4. Saeeda Bhatti: Contributed to essential reagents and tools, analysed data, and approved manuscript
5. Marry Gardiner: Contributed to essential reagents and tools, analysed data, and approved manuscript
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7. Frances Fee: Contributed to essential reagents and tools, analysed data, and approved manuscript
8. Sandra Chudleigh: Contributed to essential reagents and tools, analysed data, and approved manuscript
9. Alison Spence: Contributed to patient recruitment, analysed data, and approved manuscript
10. Wendy Taylor: Contributed to patient recruitment, analysed data, and approved manuscript
11. Nicholas Heaney: Contributed to patient recruitment, performed laboratory analysis of samples, and approved manuscript
12. Brenda Gibson: Contributed to patient recruitment, performed critical analysis of research and approved manuscript
13. Gerry Graham: Performed critical analysis of research and approved manuscript
14. Chris Halsey: Conceived research, performed research, wrote manuscript.

***Statement of conflict of interest**

Conflict of Interest Form

Manuscript Name:	Use of Quantitative Polymerase Chain Reaction (qPCR) for the Diagnosis and Monitoring of CNS leukaemia
Authors:	Yasar Mehmood Yousafzai, Amanda Smith, Linda Smith, Saeeda Bhatti, Mary Gardiner, Antony Cousins, Frances Fee, Sandra Chudleigh, Alison Spence, Wendy Taylor, Nicholas Heaney, Brenda Gibson, Gerry Graham, Chris Halsey
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Details of nature of conflict of interest:	Nothing to Declare

Reason for exclusion from analysis	Number of patients
Total number of patients enrolled	57
No leftover CSF for analysis	-4
Poor quality DNA (albumin amplification >38 CT cycles).	-7
Excluded due to unavailability of suitable primers	-5
qPCR assays excluded due to technical error during the PCR run (with insufficient DNA available to repeat)	-3
Total analysed samples	38

Supplementary table 1: Reasons for exclusion of patients from final analysis

Supplementary Materials

Characteristic		**no.**	**(%)**
Age (years)	<10	44	77.1%
	>10	13	22.9%
Sex	Male	38	66.7%
	Female	19	33.3%
Immunophenotype	BCP-ALL	51	89.4%
	T-ALL	6	10.6%
Cytogenetics	t(9;22)	1	1.9%
	MLL-rearranged	3	5.6%
	t(12;21)	10	18.5%
	High Hyperdiploid	21	36.8%
WCC	<10	28	49.1%
(x 10^9/L)	10-50	22	38.5%
	>50	7	13%
CNS disease	CNS1	50	87.7%
	TLP-	3	5.2%
	TLP+	4	7.0%
	CNS-3	0	0%
Anaemia	Hb<8g/dl	30	52.6%
	Hb>8g/dl	27	47.4%

Supplementary table 2: Demographic and clinical characteristics of enrolled patients

Supplementary table 3:

ID	Age	Sex	Diagnosis	Cytogenetics	CNS status	WCC /ul	Day 29 MRD	CSF qPCR	Outcome	Follow up (mnths)
#1	7.9	F	BCP-ALL	High hyperdiploid	1	1	High risk	No	CCR*	75
#2	17.1	M	BCP-ALL	t(9;22)	1	98.49	High risk	Yes	CCR	71
#3	11.1	F	BCP-ALL	dic(7;9) Unbalanced translocation p7 and p9	1	23.8	High risk	No	CCR	74
#4	2.8	F	BCP-ALL	High hyperdiploid	1	9.1	Risk	No	CCR	51
#5	6.4	M	BCP-ALL	DER (1,16), DER 3,+9, DEL12p	TLP +ve	3.6	Low risk	No	CCR	85
#6	15.0	F	BCP-ALL	t(9;18)	TLP - ve	167.5	High risk	No	CCR	81
#7	2.7	M	BCP-ALL	High hyperdiploid	1	82	Risk	No	CCR	71
#8	6.2	F	BCP-ALL	High hyperdiploid	1	11.7	Risk	No	CCR	54
#9	3.9	M	BCP-ALL	High hyperdiploid	1	33.2	Risk	Yes	CCR	60
#10	14.2	M	T-ALL	Normal Karyotype	1	6.4	High risk	No	CCR	87
#11	1.1	M	BCP-ALL	*MLL* rearranged	1	3.2	Low risk	Yes	CCR	55
#12	2.4	M	BCP-ALL	Normal karyotype	1	11.1	Low risk	Yes	CCR	83
#13	10.3	M	BCP-ALL	Normal karyotype	1	1.8	High risk	No	CCR	75
#14	2.5	M	BCP-ALL	High hyperdiploid	1	2	Low risk	No	CCR	65
#15	3.8	M	BCP-ALL	t(12;21)	1	10.6	Low risk	No	CCR	85
#16	6.1	M	BCP-ALL	High hyperdiploid	1	2	Low risk	Yes	CCR	62
#17	4.9	M	BCP-ALL	t(12;21)	1	52	Low risk	No	CCR	75
#18	9.1	M	T-ALL	Normal Karyotype	1	36.4	High risk	Yes	CCR	73
#19	1.1	M	BCP-ALL	High hyperdiploid	1	17.6	Risk	No	CCR	69
#20	4.9	M	BCP-ALL	High hyperdiploid	1	6.9	Risk	No	CCR	65
#21	1.7	F	BCP-ALL	High hyperdiploid	1	2.7	Risk	Yes	CCR	62
#22	2.7	M	BCP-ALL	High hyperdiploid	TLP+ ve	10.8	Low risk	Yes	CCR	81
#23	3.5	M	BCP-ALL	t(12;21)	TLP+ ve	46	Low risk	Yes	CCR	80
#24	2.3	F	BCP-ALL	High hyperdiploid	1	3.2	Risk	No	CCR	51
#25	5.5	F	BCP-ALL	High hyperdiploid	1	4.2	MRD Risk	No	Died	72
#26	5.4	F	BCP-ALL	Normal Karyotype	1	28	Low risk	Yes	CCR	80
#27	6.0	F	BCP-ALL	High hyperdiploid	1	1.1	Low risk	No	CCR	72
#28	2.5	F	BCP-ALL	t(12;21)	1	19.1	MRD risk	No	CCR	71
#29	3.7	M	BCP-ALL	t(12;21)	1	4.9	MRD Risk	No	CCR	52
#30	2.6	F	BCP-ALL	High hyperdiploid	1	6.2	Low risk	Yes	CCR	69
#31	5.4	M	BCP-ALL	t(12;21)	1	5.3	Low risk	No	CCR	85
#32	2.1	M	T-ALL	Normal Karyotype	1	56.5	Low Risk	Yes	CCR	69
#33	3.3	M	BCP-ALL	High hyperdiploid	1	1.7	Risk	No	CCR	69
#34	13.9	F	T-ALL	NA	1	28	Risk	Yes	CCR	62
#35	3.2	F	BCP-ALL	High hyperdiploid	TLP- ve	3.1	Low risk	No	CCR	53
#36	10.8	M	BCP-ALL	High hyperdiploid	TLP +ve	13.6	High risk	Yes	CCR	73
#37	2.3	M	BCP-ALL	11q23	TLP+ ve	23.4	High risk	Yes	CCR	81
#38	3.5	M	BCP-ALL	NA	1	8.1	Low Risk	No	CCR	66

Supplementary table 3: Patient characteristics and CSF qPCR status. CNS status is based on CSF cytospin analysis (CNS-1= Leukocytes <5/ul, no identifiable blasts, CNS-2 = >5 leukocytes with

identifiable blasts, CNS-3 = > 5 blasts/ul, TLP +ve = traumatic lumbar puncture with blasts, TLP -ve = traumatic lumbar puncture without blasts). MRD risk based on Molecular monitoring of minimal residual disease, CCR= Complete clinical remission, NA=Not available.

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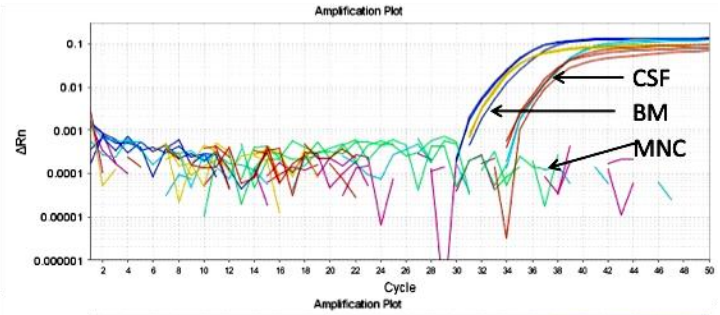
Supplementary Materials

(a)



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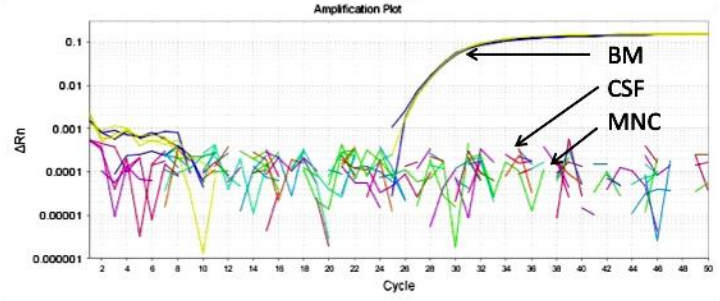

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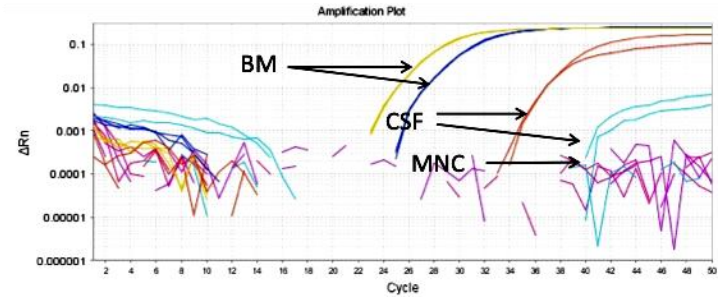

Supplementary Figure 1: qPCR amplification plots showing (a) amplification by two PCR targets, (b) no amplification seen in CSF DNA (c) amplification in 1 out of 2 PCR targets. MNC=mononuclear cell DNA from healthy controls used as a negative control.